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Herpesvirus saimiri Oncoproteins Tip and StpC Synergistically Stimulate NF-κB Activity and Interleukin-2 Gene Expression

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Saimiriine herpesvirus 2 (*Herpesvirus saimiri*) is capable of inducing lethal T-cell lymphoproliferative diseases in primates and of immortalizing human T lymphocytes *in vitro.* Two viral oncoproteins, Tip and StpC, are essential for T-cell transformation by *Herpesvirus saimiri* strains of the subgroup C, which exhibits a higher transformation potential than other subgroups of this virus. Despite the importance of these proteins, the molecular basis of their effects on T cells is poorly understood. It remains unclear how Tip and StpC affect gene expression and what is the molecular basis of their cooperation. To address these issues, we expressed Tip and StpC in T lymphoblastoid cells and assessed both their effects on and transcription factors involved in IL-2 gene expression. Our study shows that Tip and StpC cooperate to upregulate IL-2 gene expression, that their effect is mediated primarily by NF-κB and NF-AT, which is partially dependent on tyrosine phosphorylation.

Key Words: Herpesvirus saimiri; Tip; StpC; protein tyrosine kinases; interleukin-2; transcription; NF-κB; NF-AT.

INTRODUCTION

Herpesvirus saimiri is capable of inducing lethal T-cell lymphoproliferative diseases in primates and of immortalizing human T lymphocytes *in vitro* (reviewed in Broker and Fickenscher, 1999; Meinl *et al.,* 1995; Trimble and Desrosiers, 1991; Tsygankov and Romano, 1999). T cells immortalized by *H. saimiri* demonstrate a typical activated phenotype, functional biological responses including proliferation and cytokine production, interleukin-2 (IL-2) dependence, and antigen specificity (Berend *et al.,* 1993; Biesinger *et al.,* 1992; Broker *et al.,* 1993; De Carli *et al.,* 1993; Medveczky and Medveczky, 1989; Mittrucker *et al.,* 1992; Weber *et al.,* 1993). These functional characteristics make transformation of T cells by *H. saimiri* a unique experimental tool for studying T-cell malignancies and for obtaining large numbers of immortalized antigen-specific T cells.

Two open reading frames (ORFs)[2] are essential for T-cell transformation by *H. saimiri* strains of the subgroup C (Duboise *et al.,* 1998; Medveczky *et al.,* 1993), which exhibits a much higher transformation potential than do the other *H. saimiri* subgroups (Biesinger *et al.,* 1992; Desrosiers *et al.,* 1986; Medveczky *et al.,* 1989). One of these ORFs encodes StpC, a protein capable of transforming fibroblasts *in vitro* (Jung *et al.,* 1991) and causing multiple epithelial, but not lymphoid, tumors in transgenic mice (Murphy *et al.,* 1994). StpC has been shown to bind to Ras in T cells and fibroblasts and to activate Ras in fibroblasts (Jung and Desrosiers, 1995). A recently published report indicates that StpC binds to tumor necrosis factor receptor-associated factors (TRAFs) and modestly activates NF-κB when overexpressed in epithelial cells. This study also shows that a mutant form of StpC incapable of binding to TRAF2 or activating NF-κB cannot support transformation of fibroblasts and human T cells (Lee *et al.,* 1999). However, the connection between the ability of StpC to activate NF-κB in epithelial cells and the transformation potential of StpC in fibroblasts is not clear, since some forms of StpC activate NF-κB, while not transforming fibroblasts, and vice versa (Jung and Desrosiers, 1995; Lee *et al.,* 1999). Furthermore, the role of StpC interactions with Ras and TRAFs in the effects of StpC on T cells is insufficiently understood.

Unlike *stp*C, the second ORF essential for T-cell immortalization cannot transform fibroblasts (Jung *et al.,* 1991). The translation product of this ORF, Tip, binds to and is phosphorylated by Lck, an Src-family protein tyrosine kinase (PTK) (Biesinger *et al.,* 1995). It was previously shown in several experimental systems that the kinase activity of Lck is increased when this PTK interacts with Tip *in vitro* or in cells overexpressing Tip (Hartley *et al.,* 1999; Lund *et al.,* 1997b; Wiese *et al.,* 1996). Furthermore, Lck was shown to be associated with tyrosine-phosphorylated transcription factors Stat1 and Stat3 in the lysates of Tip-positive, but not Tip-negative,

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[2] Abbreviations used: EMSA, electrophoretic mobility shift assay; IL-2, interleukin-2; kDa, kilodaltons; MAb, monoclonal antibody; MSCV, murine stem cell virus; ORF, open reading frame; PTK, protein tyrosine kinase; TCR, T-cell antigen receptor; TRAF, tumor necrosis factor receptor-associated factor.

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T cells (Lund *et al.,* 1997a,b). Recent reports demonstrate that Tip is capable of upregulating Stat-dependent transcription in Jurkat cells (Hartley and Cooper, 2000). However, downregulation of Lck expression and kinase activity was shown in Jurkat-based cell lines expressing Tip as a result of retroviral transduction (Guo *et al.,* 1997; Jung *et al.,* 1995). This downregulation might be explained by the existence of a negative feedback mechanism in Jurkat cells, which prevents hyperactivation of Lck caused by Tip, to keep the activity of this important regulatory PTK under control.

Taken together, the results described herein suggest that Tip and StpC, proteins essential for *H. saimiri*-induced transformation, exert their effects on cells through interactions with cellular regulatory mechanisms. However, despite substantial advances in understanding the molecular basis of the effects of Tip and StpC, several important issues related to the mechanisms of these effects have not yet been addressed. It remains to be determined whether Tip can regulate transcription factors other than Stat. Furthermore, it remains unclear whether Tip and StpC cooperate at the level of transcriptional regulation, as they do in T-cell transformation (Duboise *et al.,* 1998; Medveczky *et al.,* 1993). Finally, contribution of protein tyrosine phosphorylation to the effects of Tip and StpC is poorly understood. To address these issues, we expressed Tip and StpC in T lymphoblastoid cells and assessed their effects on lymphokine gene expression and transcription factor activity in these cells and the role of protein tyrosine phosphorylation in these effects. We focused our study on IL-2 gene expression and transcription factors involved therein, because an increase in IL-2 production appears to play a significant role in the autocrine growth of *H. saimiri*-transformed T cells (Broker *et al.,* 1993, 1994; De Carli *et al.,* 1993; Mittrucker *et al.,* 1992, 1993). Since protein tyrosine phosphorylation is crucial for the regulation of gene expression, we also examined protein tyrosine phosphorylation in Tip- and/or StpC-expressing cells, as well as the effects of protein tyrosine phosphorylation on IL-2 gene expression and transcription factor activity. This study indicates that Tip and StpC significantly upregulate IL-2 gene expression in lymphoblastoid T cells in response to extracellular stimulation. This effect requires simultaneous expression of Tip and StpC, appears to be mediated primarily by the NF-κB and NF-AT transcription factors, and is, at least partially, dependent on protein tyrosine phosphorylation.

RESULTS

Tip and StpC facilitate, in a synergistic and protein tyrosine phosphorylation-dependent fashion, IL-2 gene expression in lymphoblastoid T cells

One major consequence of *H. saimiri*-induced transformation of T cells is a significant increase in both constitutive and inducible expression of multiple cytokines, including IL-2 and interferon-γ (Broker *et al.,* 1993, 1994; De Carli *et al.,* 1993; Mittrucker *et al.,* 1992, 1993; Weber *et al.,* 1993). We focused our study on IL-2 gene expression because production of IL-2 appears to significantly promote growth of *H. saimiri*-transformed T cells in culture (Broker *et al.,* 1994; De Carli *et al.,* 1993; Mittrucker *et al.,* 1992, 1993). Furthermore, signaling pathways and transcription factors involved in the regulation of IL-2 gene are well characterized and therefore represent a convenient system for mapping possible mechanistic links between *H. saimiri* proteins and T-cell responses. To detect IL-2 gene expression we employed several complementary approaches.

First, we analyzed the effects of Tip and StpC on IL-2 secretion by MOLT4 cells transduced with Tip and/or StpC cDNA. The cells were stimulated using various MAbs against cell surface receptors, as well as PMA and ionomycin, and the levels of IL-2 in cellular supernatants were measured. The results of these experiments demonstrate that anti-receptor MAbs, including those to CD3, CD2, CD4, and CD28, failed to stimulate IL-2 production by MOLT4 cells (data not shown). Since this failure was consistent with low densities of CD3, CD2, and CD28 on the surface of MOLT4 cell lines (data not shown), we decided to use stimulation with PMA and ionomycin to further examine the effects of Tip and StpC on these cells. This stimulation revealed a dramatic effect of Tip and StpC on IL-2 production by MOLT4 cells. While $Tip^{-}StpC^{-}$, $Tip^{+}StpC^{-}$, or $Tip^{-}StpC^{+}$ MOLT4 cells showed no IL-2 secretion either in the presence or in the absence of PMA and ionomycin, $Tip^{+}StpC^{+}$ MOLT4 cells produced substantial amounts of IL-2 in response to a mixture of PMA and ionomycin (Fig. 1).

Further analysis indicated that, despite the apparent lack of IL-2 secretion (see Fig. 1), control MOLT4 cells were capable of producing IL-2 at low levels; when supernatants were collected at 48 h after stimulation instead of typical 12 h, IL-2 was detected in the supernatants of control cells stimulated with PMA and ionomycin at approximately 100 pg/ml (data not shown). However, considering that IL-2 production by control MOLT4 cells was at least 50-fold lower than that by $Tip^{+}StpC^{+}$ cells, and that prolonged incubations were detrimental for cell viability (data not shown), we chose to collect supernatants in all further experiments at 12 h poststimulation.

To rule out the possibility that the observed effects were caused by clonal variations unrelated to Tip and StpC expression, we examined several stable clones of each cell type studied. None of the $Tip^{-}StpC^{-}$, $Tip^{+}StpC^{-}$, or $Tip^{-}StpC^{+}$ clones studied in these experiments secreted a detectable level of IL-2 in the presence or in the absence of PMA and ionomycin. By contrast, each of the three $Tip^{+}StpC^{+}$ clones studied secreted a substantial amount of IL-2 (Fig. 1).

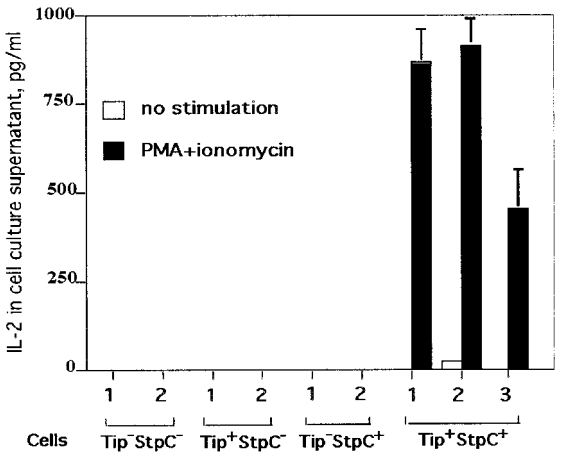

FIG. 1. Effects of Tip and StpC on IL-2 secretion by MOLT4 cells. MOLT4 cells were stimulated with PMA and ionomycin where indicated, and their supernatants were analyzed for IL-2 using sandwich ELISA. Cells expressed Tip and/or StpC, as indicated at the bottom of the figure. The numbers indicate individual clones of each type. Triplicate measurements were performed in each experiment. Means and SD are shown for one representative experiment from a total of four experiments.

To further characterize the mechanisms by which Tip and StpC upregulated IL-2 production by MOLT4 cells, we assessed the IL-2 mRNA levels in these cells using quantitative RT-PCR in the presence of a competitor, or MIMIC, fragment. MIMICs competed with the DNAs of interest for specific primers acting as internal standards in PCR reactions, allowing us to measure the concentrations of these cDNAs. The amount of IL-2 cDNA was normalized to the amount of β-actin cDNA, and the molar ratios of the IL-2 cDNA to the β-actin cDNA were determined for each sample. These experiments demonstrated that IL-2 mRNA levels in MOLT4 cells expressing no Tip or StpC, or either Tip or StpC alone, were undetectable regardless of stimulation (Fig. 2). By contrast, Tip^+StpC^+ MOLT4 cells exhibited a significant increase in their IL-2 mRNA level in response to PMA and ionomycin (Fig. 2). None of the Tip^-StpC^-, Tip^+StpC^-, or Tip^-StpC^+ clones examined in these experiments demonstrated a detectable level of IL-2 mRNA in the presence or in the absence of PMA and ionomycin, whereas each of the three Tip^+StpC^+ clones studied showed a profound increase in the amount of IL-2 mRNA, reaching approximately 0.1 of the β-actin mRNA level (data not shown). Therefore, the effects of Tip and StpC on the level of IL-2 mRNA in MOLT4 cells correlated well with their effects on IL-2 secretion by these cells (see Fig. 1), indicating that Tip and StpC facilitated IL-2 production at the level of IL-2 mRNA.

Previous findings indicating the crucial role of protein tyrosine phosphorylation in IL-2 gene expression (reviewed in DeFranco, 1995; Howe and Weiss, 1995; Sefton and Taddie, 1994; Zenner *et al.,* 1995), as well as the ability of Tip to facilitate protein tyrosine phosphorylation in T cells (Lund *et al.,* 1997b; Wiese *et al.,* 1996), prompted us to assess the effect of PTK inhibitors on the Tip/StpC-facilitated IL-2 gene expression in MOLT4 cells. These experiments showed that the PMA/ionomycin-induced increases in IL-2 secretion and mRNA level were significantly reduced in a concentration-dependent fashion by genistein and herbimycin A, PTK inhibitors of broad specificity (Fig. 3A and data not shown). Likewise, PP1, an inhibitor believed to be specific for Src-family PTKs, such as Lck and Fyn (Hanke *et al.,* 1996), decreased IL-2 secretion by Tip^+StpC^+ MOLT4 cells (Fig. 3B). Genistein, herbimycin A, and PP1 caused a decrease in IL-2 transcription and secretion not only in MOLT4 cells but also in *H. saimiri*-transformed T cells (data not shown). Unlike PTK inhibitors, several other potential inhibitors tested in these experiments, such as rapamycin at 100 ng/ml and wortmannin at 1 μM, did not reduce IL-2 gene expression in MOLT 4 cells. As expected, cyclosporin A (500 ng/ml) and staurosporin (5 μM), potent inhibitors of Ca^{2+}-dependent signaling and protein kinase C, respectively, completely abrogated IL-2 gene expression in this system (data not shown).

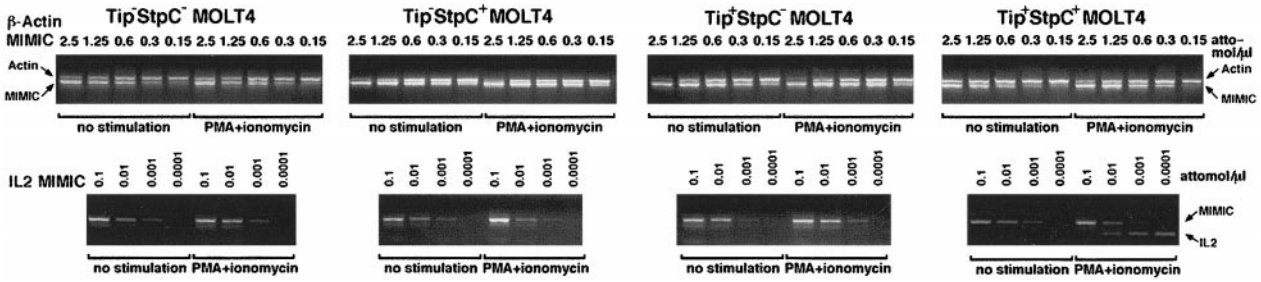

FIG. 2. Effects of Tip and StpC on IL-2 mRNA levels in MOLT4 cells. MOLT4 cells were stimulated with PMA and ionomycin where indicated, harvested, and used for RNA preparation. Quantitative DNA amplification in the presence of MIMIC fragments is shown for MOLT4 cells expressing Tip and/or StpC, as indicated at the figure. Concentrations of MIMIC fragments corresponding to IL-2 and β-actin are indicated at the top of each panel. The positions of target cDNAs and MIMICs are indicated by arrows. In each experiment of this type, a 10-fold titration (as shown) was followed by a twofold titration to more precisely measure the amount of specific cDNAs, and the ratios between cDNAs corresponding to IL-2 and β-actin transcripts were calculated. One representative experiment from a total of five experiments is shown.

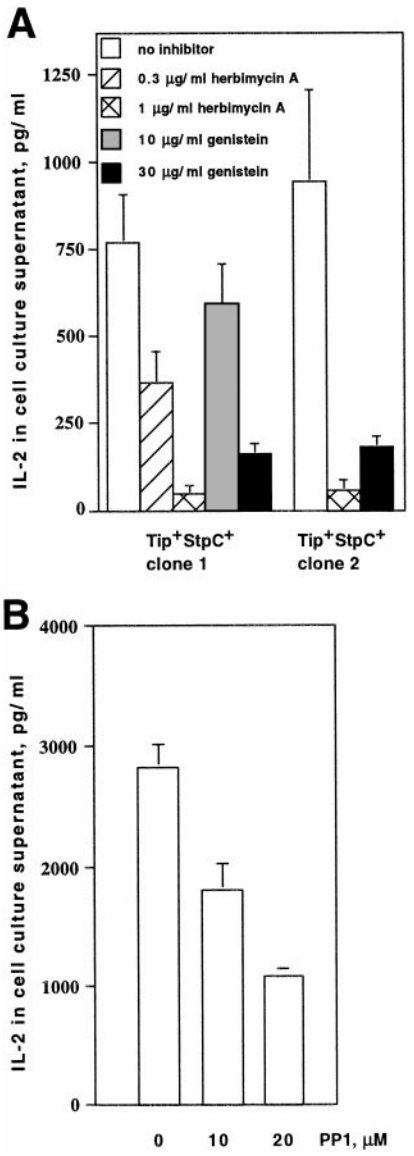

FIG. 3. Effect of PTK inhibitors on IL-2 production by MOLT4 cells. (A) Two individual clones of Tip^+StpC^+ MOLT4 cells were stimulated with PMA and ionomycin in the absence or in the presence of PTK inhibitors, herbimycin A and genistein, at the concentrations indicated. The amount of IL-2 in cellular supernatants was determined using ELISA. Tip^+StpC^+ cells did not secrete IL-2 in the absence of stimulation or in the presence of PMA alone or ionomycin alone (see Fig. 1 and text). Triplicate measurements were performed in each experiment. Means and SD for one representative experiment from a total of three experiments are shown. (B) Tip^+StpC^+ MOLT4 cells were treated with PP1 and stimulated with PMA and ionomycin, and their supernatants were analyzed for IL-2 as described in (A). Means and SD for one representative experiment from a total of three experiments are shown.

Considering that Ras is a potential target of StpC in T cells (see Introduction), we attempted to elucidate the role of Ras in the observed increase in IL-2 production by Tip^+StpC^+ MOLT4 cells. Tip^+StpC^+ MOLT4 cells were electroporated with an expression construct encoding wild-type or inactive N17 Ras, or the corresponding empty vector, along with the EGFP-C1 plasmid. Cells fluorescing green were selected and analyzed for IL-2 production. In these experiments, Tip^+StpC^+ MOLT4 cells transfected with N17 Ras showed a very high level of N17 Ras expression, dramatically exceeding that of endogenous Ras, but no decrease in IL-2 production as compared to that of Tip^+StpC^+ cells transfected with the empty vector (Fig. 4). It appeared impossible to compare the effects of overexpression of N17 Ras and wild-type Ras, because the latter caused massive cell death in our system (data not shown).

Since the effects of Tip and StpC on MOLT4 cells were only seen following stimulation with PMA and ionomycin, we next examined the effects of these proteins on Jurkat cells, which express multiple surface receptors at higher levels than do MOLT4 cells. First, Jurkat cells simultaneously expressing Tip and StpC demonstrated a modest, but detectable, increase in IL-2 production in response to suboptimal concentrations of PMA and ionomycin over the level corresponding to vector control Jurkat cells (data not shown). Furthermore, production of IL-2 in response to anti-CD3 and anti-CD3 plus anti-CD28 MAbs was significantly upregulated in Tip^+StpC^+ Jurkat cells. Thus, anti-CD3-induced IL-2 production was fourfold higher in Tip^+StpC^+ cells than in vector control (Tip^-StpC^-) cells (Fig. 5). Therefore, these experiments demonstrated that Tip and StpC facilitated IL-2 production not only induced by PMA and ionomycin but also triggered by ligation of surface receptors. However, the Tip/StpC-facilitated increase in receptor-mediated IL-2 production in Jurkat cells was significantly less profound than the Tip/StpC-facilitated increase in PMA/ionomycin-induced production of IL-2 in MOLT4 cells. This finding, together with the fact that cloning efficiency of Tip^+StpC^+ Jurkat cells was low compared to that of Tip^+StpC^+ MOLT4 cells, thus preventing us from obtaining multiple individual clones of Tip^+StpC^+ Jurkat and restricting our work to the polyclonal Tip^+StpC^+ Jurkat cell line, prompted us to carry out all our further experiments in MOLT4 cells.

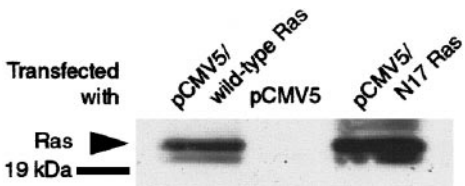

FIG. 4. Transient expression of wild-type and N17 Ras in Tip^+StpC^+ MOLT4 cells. Tip^+StpC^+ MOLT4 cells were electroporated in the presence of pCMV5 plasmids encoding wild-type or N17 Ras or the empty pCMV5 vector, harvested, and lysed. The lysates were loaded on a 12% SDS–PAGE as indicated at the top of the figure, separated, transferred to nitrocellulose, and probed with anti-Ras. Protein bands were visualized using chemiluminescence. A prolonged exposure revealed a band of the endogenous Ras. The positions of a 19-kDa protein standard and Ras are shown by a horizontal bar and an arrowhead, respectively. One representative experiment from a total of two is shown.

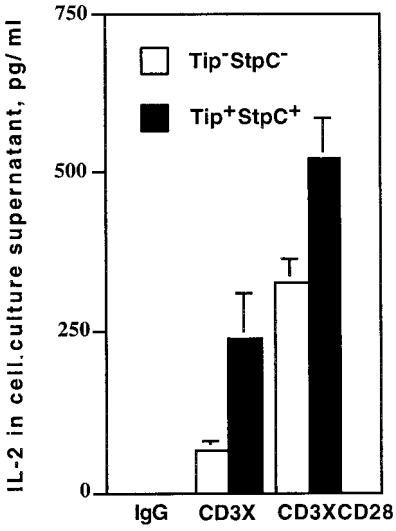

FIG. 5. Effects of Tip and StpC on IL-2 secretion by Jurkat cells. Tip^+StpC^+ and Tip^-StpC^- (vector control) Jurkat cell lines were stimulated with anti-surface receptor MAbs or a control IgG as indicated, and their supernatants were analyzed for IL-2 using sandwich ELISA. Triplicate measurements were performed in each experiment. Means and SD are shown for one representative experiment from a total of two experiments.

Tip and StpC upregulate activity of transcription factors involved in IL-2 gene expression

A significant increase in the level of IL-2 mRNA in Tip^+StpC^+ MOLT4 suggested that Tip and StpC might activate IL-2 transcription. To directly assess the activity of IL-2 promoter/enhancer in MOLT4 cells, we transiently transfected MOLT4 cells with reporter constructs, in which transcription of firefly luciferase was driven by the full-length IL-2 promoter/enhancer. These experiments demonstrated that Tip^+StpC^+ MOLT4 responded to PMA/ionomycin stimulation by a substantial increase in IL-2 promoter/enhancer-controlled luciferase production, whereas vector control cells and cells expressing Tip or StpC alone exhibited no increase in IL-2 promoter/enhancer-controlled luciferase production (Fig. 6A). This result was consistent with the observed increase in IL-2 mRNA and protein levels in MOLT4 cells (see Figs. 1–3) and argued that the effects of Tip and StpC on IL-2 gene expression were directly mediated by transcription.

To elucidate the mechanisms by which Tip and StpC upregulate transcription of the IL-2 gene, we analyzed effects of these proteins on specific DNA response elements, AP-1, NF-AT, and NF-κB, previously determined to be crucial for the regulation of IL-2 gene expression (reviewed in Rothenberg and Ward, 1996; Serfling *et al.,* 1995). The corresponding reporter constructs were similar to the full-length IL-2 promoter/enhancer plasmid, but contained oligomerized individual DNA response elements fused to the minimal IL-2 promoter. The experiments with these constructs showed that their transcriptional activity was sensitive to extracellular stimulation with PMA and ionomycin, although to varying extents (Figs. 6B and 6C and data not shown). An increase in NF-AT-controlled transcription in MOLT4 cells induced by PMA and ionomycin was dramatically facilitated by Tip. StpC also facilitated NF-AT-controlled transcription, but to a lower extent. Simultaneous expression of Tip and StpC facilitated NF-AT activation to a level comparable to that corresponding to Tip alone (Fig. 6B).

Effects of Tip and StpC on NF-κB-controlled transcription significantly differed from their effects on NF-AT. First, an increase in NF-κB-controlled transcription induced by PMA and ionomycin was dramatically aug-

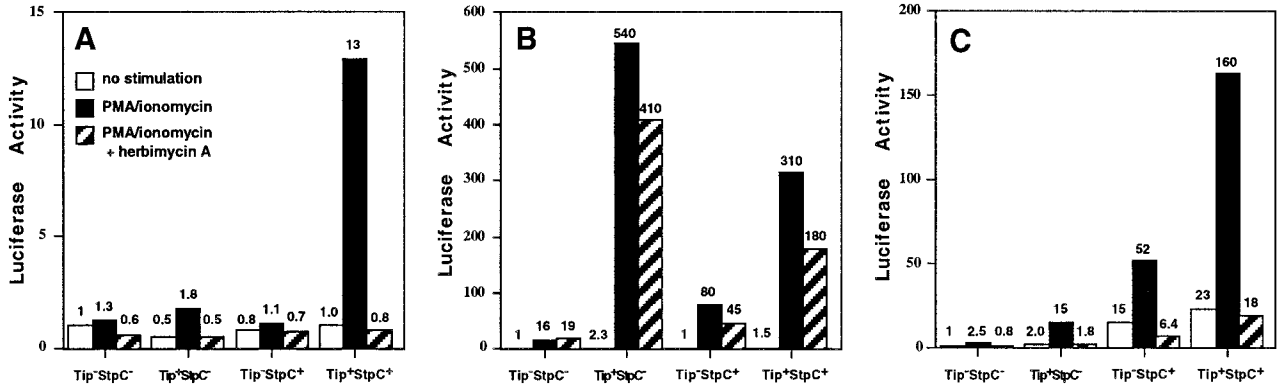

FIG. 6. Effects of Tip and StpC on transcriptional activity of the full-length IL-2 promoter/enhancer and its DNA response elements in MOLT4 cells. MOLT4 cells expressing Tip and/or StpC as indicated at the bottom of each panel, were transfected with a *Renilla* luciferase internal control plasmid and a firefly luciferase reporter construct: (A) the full-length IL-2 promoter/enhancer, (B) NF-AT, (C) NF-κB. Cells were treated as indicated and lysed. Firefly luciferase activities were normalized using the corresponding *Renilla* luciferase activities. Normalized firefly luciferase activity in nonstimulated Tip^-StpC^- cells was assigned the value of 1.0 for each reporter construct. Normalized luciferase activities for a representative experiment from a total of at least three independent experiments for each panel are shown. All the cell lines used in the experiments were also examined for the ability to express firefly luciferase from the pGL3 control plasmid containing SV40 promoter and enhancer elements (Promega) and were found to be equally capable of this expression (data not shown).

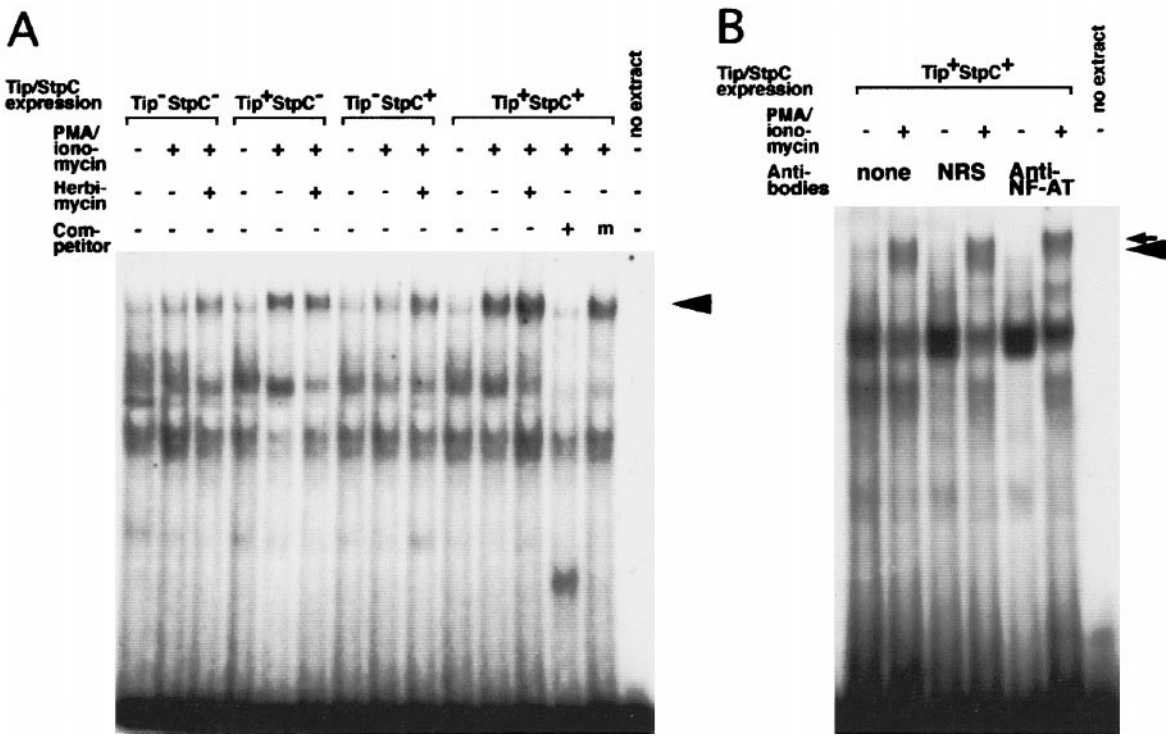

FIG. 7. Effects of Tip and StpC on NF-AT DNA-binding activity in MOLT4 cells. MOLT4 cells expressing Tip and/or StpC were stimulated as indicated at the top of each panel. Their nuclear proteins were obtained and analyzed in binding assays with a ^{32}P-labeled DNA probe corresponding to the NF-AT binding site. Herbimycin was added to the cells as indicated at the top of (A). An anti-NF-AT antibody or normal rabbit serum (NRS) was added to binding mixtures as indicated at the top of (B). Unlabeled wild-type (+) or mutant (m) competitors were added at a 30-fold excess. Specific DNA–protein complexes are shown by arrowheads. Supershifted complex is shown by an arrow. One representative experiment from a total of at least three experiments for each panel is shown.

mented by StpC, whereas Tip exhibited only a modest effect. Second, the simultaneous expression of Tip and StpC facilitated NF-κB-controlled transcription to a much higher extent than did the expression of either Tip or StpC alone. Third, expression of StpC with or without Tip significantly elevated NF-κB-controlled transcription in unstimulated cells (Fig. 6C).

Unlike NF-AT and NF-κB, AP-1 showed a high level of constitutive activity in MOLT4 cells and only a modest further increase in response to PMA and ionomycin. No significant differences in AP-1 activity were observed between control cells and cells expressing Tip and/or StpC (data not shown).

Since IL-2 gene expression in Tip^+StpC^+ MOLT4 was found to be sensitive to PTK inhibitors (see Fig. 3), we assessed the effect of herbimycin A on transcriptional activities of all the reporter constructs utilized in our experiments. This analysis revealed a significant difference in sensitivities of their enhancer elements to PTK inhibition. A PMA/ionomycin-induced increase in the transcriptional activity of full-length IL-2 promoter/enhancer or NF-κB response element was completely suppressed by herbimycin, whereas those of NF-AT and AP-1 were inhibited by less than 50% (Fig. 6 and data not shown).

To analyze the ability of Tip and StpC to activate transcription factors involved in IL-2 gene expression using an independent approach, we assessed binding of MOLT4 nuclear proteins to DNA probes corresponding to the AP-1, NF-AT, and NF-κB binding sites using EMSA. These experiments showed that the NF-AT-specific probe did not appreciably bind to proteins of nuclear extracts from the vector control MOLT4 cells, either unstimulated or stimulated with PMA and ionomycin (Fig. 7A). However, stimulation-induced NF-AT binding was significantly increased in MOLT4 cells expressing Tip alone or Tip and StpC simultaneously, but not in the cells expressing StpC alone (Fig. 7A). The NF-AT binding was increased in Tip^+StpC^- MOLT4 and Tip^+StpC^+ MOLT4 to a similar level, indicating, in agreement with the data obtained using reporter constructs (see Fig. 6B), that Tip alone was sufficient to activate NF-AT binding. A discrepancy between the ability of StpC to facilitate NF-AT-controlled expression of luciferase (Fig. 6B) and its failure to facilitate binding of proteins to a NF-AT-specific probe (Fig. 7A) could be explained either by a higher sensitivity of luciferase assays or by the involvement of mechanisms unrelated to NF-AT-specific DNA–protein binding, or by a combination of these two factors.

The observed binding to the NF-AT probe was specific, since it was inhibited by the identical unlabeled probe, but not by the probe containing mutations rendering it

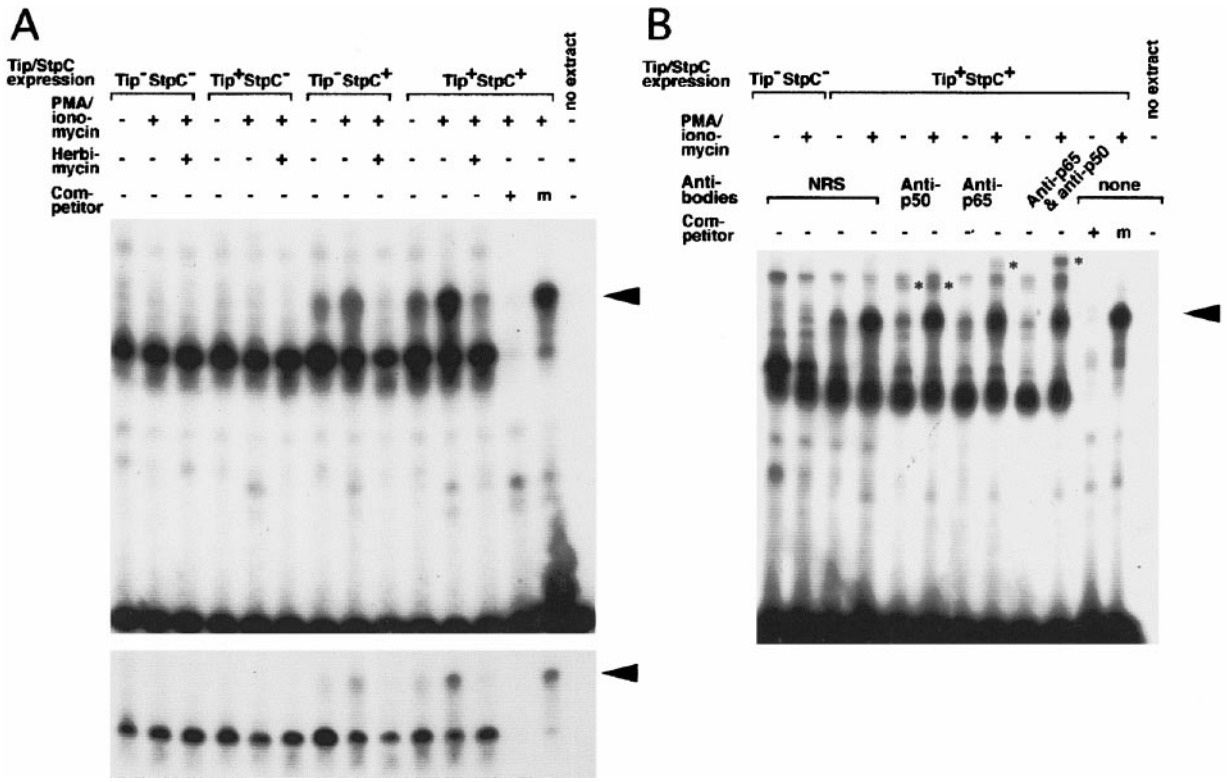

FIG. 8. Effects of Tip and StpC on NF-κB DNA-binding activity in MOLT4 cells. MOLT4 cells expressing Tip and/or StpC were stimulated as indicated at the top of each panel. Their nuclear proteins were obtained and analyzed in binding assays with a ^{32}P-labeled DNA probe corresponding to the NF-κB binding site. Herbimycin was added to the cells as indicated at the top of (A). The top and the bottom panels in (A) demonstrate, respectively, a long and a short exposure of the same gel. Antibodies to NF-κB proteins or normal rabbit serum (NRS) were added to binding mixtures as indicated at the top of (B). Unlabeled wild-type (+) or mutant (m) competitors were added at a 30-fold excess. Specific DNA–protein complexes are shown by arrowheads. Supershifted complexes are shown by asterisks. One representative experiment from a total of at least three experiments for each panel is shown.

binding-incompetent (Fig. 7A). To further elucidate the specificity of this binding, we performed EMSA in the presence of anti-NF-AT and observed a supershift in the presence of this antibody, but not in the presence of preimmune serum (Fig. 7B). This result further argued that NF-AT was present in the detected DNA–protein complex. We then assessed NF-AT binding in cell lysates from various individual MOLT4 clones and demonstrated very similar levels of NF-AT binding in several Tip^+StpC^+ clones (data not shown). This experiment ruled out that the results shown in Fig. 7 could be a consequence of random clonal variations.

A set of similar experiments carried out to analyze NF-κB binding showed no appreciable binding in the vector control cells or the Tip-only cells, either stimulated or unstimulated. In contrast, NF-κB binding was detectable in Tip^-StpC^+ MOLT4 in the absence of stimulation and further increased following stimulation with PMA and ionomycin (Fig. 8A). Furthermore, nuclear extracts of Tip^+StpC^+ MOLT4 stimulated with PMA and ionomycin showed a significant increase in NF-κB binding activity compared to those of Tip^-StpC^+ MOLT4 (Fig. 8A). Therefore, the findings of EMSA experiments with the NF-κB probe were consistent with the results obtained using the NF-κB reporter construct, except that an increase in NF-κB transcriptional activity in MOLT4 cells expressing Tip alone was detected using luciferase reporter assays (Fig. 6C), but not EMSA (Fig. 8A). This finding, like that for NF-AT, could be explained either by a higher sensitivity of reporter assays or by the involvement of mechanisms unrelated to specific DNA–protein binding to the observed increase in reporter activity, or by a combination of both factors.

Specificity of NF-κB binding was confirmed using unlabeled wild-type and mutated NF-κB probes (Fig. 8). To further characterize specificity of the observed interactions, we carried out supershift experiments using the approach described above for NF-AT. These experiments identified p50 and p65 as components of the NF-κB complex in Tip^+StpC^+ MOLT4 cells (Fig. 8B). To rule out the possibility that the results shown in Fig. 8 were a consequence of random differences between individual clones, we assessed levels of NF-κB binding in several Tip^+StpC^+ MOLT4 clones and found these levels to be very similar (data not shown).

AP-1 binding activity of MOLT4 nuclear proteins was detectable in nonstimulated cells and remained essentially unaffected by expression of Tip and StpC (data not

shown). These findings were consistent with the results obtained using the AP-1 reporter construct (see above). The failure of Tip and StpC to affect AP-1 binding together with a high level of AP-1 binding in nonstimulated MOLT4 cells argue against a substantial involvement of AP-1 in mediating the observed effects of Tip and StpC on IL-2 gene expression.

The results of EMSA experiments demonstrated, in agreement with the data obtained using reporter constructs, that herbimycin A did not inhibit either NF-AT or AP-1 binding in MOLT4 cells (Fig. 7A and data not shown). In contrast, NF-κB binding was dramatically inhibited by herbimycin A in both $StpC^+$ MOLT4 and Tip^+StpC^+ MOLT4 cells (Fig. 8A). This finding is consistent with the corresponding reporter construct results (see Fig. 6), indicating that the effect of Tip and StpC on NF-κB activity is PTK-dependent.

Since the level of IL-2 mRNA is also affected by the life span of IL-2-producing T cells, the observed effect of Tip and StpC on IL-2 gene expression may be mediated, at least in part, by their antiapoptotic effect on MOLT4 cells. To address this issue, we examined the effect of simultaneous expression of Tip and StpC on viability and apoptosis of MOLT4 cells. These experiments showed that the number of viable cells was somewhat decreased in cultures of Tip^+StpC^+ MOLT4 cells compared to those of Tip^-StpC^- vector control cells, whereas the amount of cytoplasmic DNA–histone complexes indicative of apoptosis was slightly increased in Tip^+StpC^+ MOLT4 cells compared to that in vector control cells (data not shown). These results indicated that an increase in IL-2 gene expression caused by Tip and StpC was not mediated by an increase in T-cell resistance to stimulation-induced apoptosis. This result was consistent with a report indicating that *H. saimiri*-induced transformation did not increase resistance of T cells to apoptosis (Kraft *et al.,* 1998).

DISCUSSION

Despite the established role of Tip and StpC in *H. saimiri*-induced T-cell transformation, little is known about the molecular mechanisms of their effects. This is, at least in part, the result of difficulties in separating the partial effects of Tip and StpC in *H. saimiri*-transformed T cells. Furthermore, it is important to separate the effects of Tip and StpC on T cells from possible effects of other *H. saimiri* gene products, such as a IL-17-like cytokine (Fossiez *et al.,* 1996), a homolog of the IL-8 receptor (Albrecht *et al.,* 1992; Yao *et al.,* 1995), regulators of gene expression (Nicholas *et al.,* 1988; Whitehouse *et al.,* 1998a,b), a homolog of the mouse mammary tumor virus superantigen (Albrecht *et al.,* 1992; Knappe *et al.,* 1997), a cyclin D3 homolog (Albrecht *et al.,* 1992; Jung *et al.,* 1994), and a *bcl-2* homolog (Derfuss *et al.,* 1998; Nava *et al.,* 1997). Although constitutive expression of genes other than *tip* and *stp*C in *H. saimiri*-transformed human T cells has not been shown, their effects on T cells cannot be ruled out. Therefore, we chose expression of isolated Tip and/or StpC cDNAs in lymphoblastoid cells as an experimental system in which effects of Tip and StpC can be separated from each other, as well as from the effects of other *H. saimiri* proteins. Although this system does not allow for assessing the ability of Tip and StpC to transform T cells, this shortcoming is compensated by its advantages for analyzing effects of Tip and StpC on signal transduction and gene expression, the deciphering of which is crucial for the understanding of interactions between *H. saimiri* and cells transformed by this virus.

Although it was previously shown that both Tip and StpC are essential for *H. saimiri*-induced transformation (Duboise *et al.,* 1998; Medveczky *et al.,* 1993), the molecular basis of the effects of Tip and StpC on T-cell responses remains poorly understood. In this report, we demonstrate that simultaneous expression of Tip and StpC in Jurkat and MOLT4 cells significantly facilitates IL-2 gene expression induced by extracellular stimulation (Figs. 1–3, 5). Our results indicate that the effect of the simultaneous expression of Tip and StpC on IL-2 production in Jurkat cells is substantially less profound than this effect in MOLT4 cells. Although the reasons for this disparity are not entirely clear, the difference in the levels of stimulation-induced IL-2 production between wild-type Jurkat cells and MOLT4 cells may play a role (Figs. 1 and 5); the low level of IL-2 production by MOLT4 cells may highlight the effect of Tip and StpC, whereas the high level of IL-2 production by Jurkat cells may mask these effects.

The results presented in our report identify the IL-2 gene as a target for the cooperative effect of Tip and StpC, independent of any other *H. saimiri* proteins. This effect of Tip and StpC is clearly synergistic, since neither Tip nor StpC alone is able to facilitate IL-2 production (Fig. 1). Our results are consistent with the previously published data that human T cells infected with wild-type *H. saimiri,* but not with *H. saimiri*-deletion mutants lacking either Tip or StpC, upregulate expression of the IL-2 gene (Chou *et al.,* 1995). The synergism between Tip and StpC observed in our study appears to be the result of the differential enhancement of transcription factor activity by these proteins. Tip alone is capable of activating NF-AT in MOLT4 cells to a significantly higher extent than is StpC alone (Figs. 6B and 7). By contrast, StpC alone can activate NF-κB in these cells to a much higher extent than can Tip alone (Figs. 6C and 8). Since both NF-AT and NF-κB should be activated to upregulate IL-2 gene transcription (reviewed in Rothenberg and Ward, 1996; Serfling *et al.,* 1995), it is apparent that both Tip and StpC are required to cause the observed dramatic increase in IL-2 gene expression in the T cells studied. Furthermore, we showed that profound synergism between Tip and

StpC also exists at the level of activation of a single transcription factor, NF-κB. Although Tip alone activates NF-κB to a very modest extent, it significantly enhances the StpC-induced activation of NF-κB (Figs. 6C and 8). This effect is likely to be another reason for the synergism between Tip and StpC in upregulating IL-2 gene expression.

AP-1 activity remains unaffected by either Tip or StpC. The failure of Tip and StpC to facilitate AP-1 activation appears to be irrelevant to the observed increase in IL-2 gene expression, since constitutive AP-1 activity in MOLT4 cells is high and can be increased further by PMA and ionomycin, albeit to a modest extent, regardless of the presence of Tip or StpC. It remains to be elucidated whether transcription factors other than NF-AT, NF-κB, and AP-1, such as EGR-1 (Skerka *et al.,* 1995), play a role in the effects of Tip and StpC on IL-2 gene expression.

It was recently shown that, through its ability to interact with Stat transcription factors, Tip causes an increase in Stat-dependent transcription in Jurkat cells expressing the SV-40 large T antigen, including a twofold increase in c-*fos* transcription (Hartley and Cooper, 2000). This Tip-facilitated increase in Stat activity is likely to contribute to the overall effect of Tip on T cells. It may also contribute to the activation of NF-AT in our system that is induced by Tip in a StpC-independent fashion, since c-Fos is a functional component of the NF-AT transcription complex (reviewed in Rao, 1994). However, the Tip-mediated activation of Stat may not exert a direct effect on IL-2 promoter/enhancer, which lacks Stat-specific response elements (reviewed in Rothenberg and Ward, 1996; Serfling *et al.,* 1995). Moreover, the effect of Tip and StpC on NF-κB described in our study is most likely independent of Stat activity, since no direct connection between these systems of transcriptional regulation has yet been established. Another recent report indicates that expression of StpC in an epithelial cell line modestly stimulates NF-κB, as shown using a NF-κB-sensitive reporter construct (Lee *et al.,* 1999). Besides these findings, little is known about the effects of Tip and StpC on transcription. In particular, no information is available regarding interactions of Tip and StpC at the level of transcriptional regulation and gene expression. Our report is the first demonstration of a profound synergistic effect of Tip and StpC on DNA binding and transcriptional activity of NF-κB, as well as IL-2 transcription, in cells expressing these viral oncoproteins in the absence of other *H. saimiri* proteins.

The synergistic activation of NF-κB by Tip and StpC in MOLT4 cells appears to be significantly reduced by herbimycin A, a potent inhibitor of PTKs (Figs. 6C and 8). Therefore, the effect of Tip and StpC on NF-κB is, at least partially, dependent on PTK activity. This conclusion is in agreement with the ability of PTK inhibitors, including PP1, which is specific for Src-family PTKs, to reduce Tip/StpC-facilitated IL-2 gene expression and IL-2 promoter/enhancer activity (Figs. 3 and 6A). Inhibition of IL-2 production by PP1 (Fig. 3B) argues that Lck may be involved in the observed effects of Tip and StpC on MOLT4 cells. This notion is consistent with observations made in multiple experimental systems that the kinase activity of Lck and tyrosine phosphorylation of cellular protein is increased in cells expressing Tip and that the activity of Lck is elevated when this PTK interacts with Tip *in vitro* (Hartley *et al.,* 1999; Lund *et al.,* 1997b; Wiese *et al.,* 1996). Considering these results, downregulation of Lck activity in Jurkat cells that were transduced with a retroviral vector to express Tip (Guo *et al.,* 1997; Jung *et al.,* 1995) is unlikely to be an immediate result of the interactions between Tip and Lck but, rather, is a consequence of the negative feedback mechanism, which prevents hyperactivation of Lck, an important regulatory PTK. It is unclear whether such a mechanism is engaged in *H. saimiri*-transformed cells, although the apparent lack of significant downregulation of Lck in human T cells transformed by *H. saimiri* (Biesinger *et al.,* 1995; Lund *et al.,* 1997b; Wiese *et al.,* 1996) argues against this hypothesis.

However, PP1 shows no absolute specificity for Lck (Hanke *et al.,* 1996), and the observed inhibition of IL-2 production by PP1 appears to be incomplete (Fig. 3B). Therefore, participation of PTKs other than Lck in the observed effects of Tip and StpC cannot be ruled out. Our results, demonstrating that T-cell responses to PMA and ionomycin facilitated by Tip and StpC are PTK-dependent, suggest that crucial tyrosine phosphorylation targets are located either downstream of targets of PMA and ionomycin or in PMA/ionomycin-insensitive signaling pathways. The nature of these targets and the mechanisms by which Tip increases their tyrosine phosphorylation in MOLT4 and Jurkat cells remains to be elucidated further. It is possible that PTKs acting downstream of phosphatydilinositol-3′ kinase and phospholipase Cγ (August *et al.,* 1997; Lev *et al.,* 1995; Li *et al.,* 1997; Okazaki *et al.,* 1997) may be involved in the effects of Tip and StpC. It remains intriguing and unclear why, despite the importance of protein tyrosine phosphorylation for the overall effect of Tip and StpC on IL-2 production, the effect of Tip on NF-AT activation in MOLT4 cells was shown to be insensitive to PTK inhibitors (Figs. 6B and 8). This result indicates that Tip activates NF-AT in a PTK-independent fashion, thus requiring further investigation.

The notion that the synergistic effect of Tip and StpC on NF-κB activity and, ultimately, on IL-2 production is likely to be mediated by signaling molecules that are located well downstream in signal transduction pathways is further supported by our current results, demonstrating that dominant-negative Ras does not inhibit IL-2 production increased by Tip and StpC. Therefore, the ability of StpC to bind to and activate Ras (Jung and Desrosiers, 1995) is unlikely to be related to the contri-

bution of StpC to an increase in IL-2 production in our experimental system. Thus, molecular targets of StpC and mechanisms by which it participates in the observed upregulation of IL-2 production remain to be elucidated.

Overall, the observed effects of Tip and StpC on cellular signaling and gene expression in MOLT4 cells agree with the idea that a critical role is played by these proteins in *H. saimiri*-induced transformation. Indeed, it was previously shown that IL-2 promotes TCR-independent growth of *H. saimiri*-transformed T cells in an autocrine fashion (Broker *et al.,* 1994; De Carli *et al.,* 1993; Mittrucker *et al.,* 1992, 1993). TCR-independent production of IL-2 by *H. saimiri*-transformed T cells appears to be triggered by the constant CD2-mediated stimulation of these cells induced by cell–cell contacts (De Carli *et al.,* 1993; Fickenscher *et al.,* 1997; Mittrucker *et al.,* 1992; Weber *et al.,* 1993). Supporting this notion, the effects of Tip and StpC on IL-2 gene expression in MOLT4 cells are stimulation-dependent.

It should be noted, however, that the T-cell systems used in this study have certain limitations resulting from the lack of expression of *H. saimiri* genes other than *tip* and *stp*C. Although the activation of transcription factors and IL-2 gene expression by Tip and StpC observed in MOLT4 and Jurkat cells is likely to mirror the effects of these *H. saimiri* proteins on multiple types of T cells, these findings are unlikely to fully describe the contribution of Tip and StpC to the biology of *H. saimiri,* in which the interactions of Tip and StpC with other *H. saimiri* proteins are likely to be important.

Finally, the observed effects of Tip and StpC on transcription factors are likely to affect multiple genes. For example, it was recently shown that Tip and StpC dramatically affect HIV replication in MOLT4 cells (Henderson *et al.,* 1999). Therefore, Tip and StpC may be interesting not only for their role in *H. saimiri*-induced transformation, but also as potential modifiers of various T-cell responses.

MATERIALS AND METHODS

Plasmids and transfection

The pCRII and MSCV vectors containing cDNAs of Tip and StpC were described previously (Merlo *et al.,* 1998). Wild-type Ras and its N17 inactive form were expressed using pCMV5-based vectors (kindly provided by Dr. D. Dhanasekaran, Temple University, Philadelphia, PA). Cells were cotransfected, where indicated, with an expression plasmid encoding green fluorescent protein (EGFP-C1; Clontech, Palo Alto, CA). Expression plasmids were transfected into T cells using electroporation in 500 μl of 2×10^7/ml cell suspension in the Opti-MEM medium (Gibco/BRL, Grand Island, NY) at 250 V, 950 μF, in the presence of 50 μg expression vector.

Cell lines and clones

Human MOLT4 and Jurkat lymphoblastoid T cells and hybridomas producing MAbs to CD2, CD3, CD4, and CD8 (OKT11, OKT3, OKT4, and OKT8, respectively) were obtained from ATCC (Rockville, MD). MOLT4 and Jurkat cells were transduced to express Tip and/or StpC by retroviral gene transfer as described previously (Merlo *et al.,* 1998). Cells transduced with the puromycin *N*-acetyltransferase gene (*pac*)-containing vectors were selected using puromycin (Sigma, St. Louis, MO). Cells transduced with the neomycin phosphotransferase gene (*neo*)-containing vectors were selected with G418 (Mediatech, Herndon, VA). The clones were generated by limiting dilution and then analyzed for integration of the corresponding cDNAs into their genomic DNA and for the presence of protein products of *tip* and *stp*C as described previously (Merlo *et al.,* 1998). Multiple individual clones of transduced MOLT4 cells were used. Polyclonal cell lines of transduced Jurkat cells were utilized, since few individual clones of transduced Jurkat cells were generated. T cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, L-glutamine, antibiotics, and HEPES (Gibco/BRL), as well as puromycin and/or G418 where indicated. Where indicated, cells were analyzed or sorted using an EPICS flow cytometer (Coulter, Fullerton, CA). Cells were stained for flow cytometry with MAbs to CD2, CD3, CD4, CD8, or CD28 described in the previous section followed by FITC-labeled goat anti-mouse IgG (Cappel, Durham, NC) using protocols described previously (Tsygankov *et al.,* 1992).

Activation of cells

T cells were harvested and resuspended in RPMI 1640 supplemented with 20 mM HEPES at a density of either 2×10^7/ml or 1×10^6/ml for biochemical and gene expression experiments, respectively, unless indicated otherwise. To study IL-2 transcription and transcription factor activity, cells were stimulated with PMA and ionomycin (both from Sigma) at final concentrations of 50 ng/ml and 1 μM, respectively, at 37°C for 4 h. To study IL-2 secretion, cells were stimulated under the same conditions, but supernatants were collected at 12 h after stimulation unless indicated otherwise. To study IL-2 production in response to stimulation through surface receptors, cells were incubated in culture plates coated with anti-receptor MAbs. The plates were precoated with goat anti-mouse IgG (Cappel) at a concentration of 10 μg/ml overnight and then coated with appropriate MAbs at 1 μg/ml for 2 h. The OKT3, OKT4, OKT8, and OKT11 MAbs were prepared from hybridoma supernatants using protein A–Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's protocol. Anti-CD28 MAb was purchased from Pharmingen (San Diego, CA). Genistein or herbimycin A (both from BioMol

Research Laboratories, Plymouth Meeting, PA) was added 15 min or 15 h prior to stimulation, respectively. Staurosporin, cyclosporin A, rapamycin, and PP1 (all from BioMol Research Laboratories) and wortmannin (Alexis, San Diego, CA) were added 30 min prior to stimulation.

Preparation of RNA and cDNA

Total RNA was isolated from T cells using the RNAzol B reagent (Tel-Test, Friendswood, TX) according to the manufacturer's recommendations. Briefly, cells were mixed first with RNAzol and then with chloroform. The samples were centrifuged at 12,000 *g* for 15 min at 4°C and the aqueous layers containing RNA were collected. RNA was precipitated with isopropanol and then washed with ethanol. The obtained RNA was denatured at 75°C and a 5-μg aliquot was added to the reaction mixture containing 10–20 units avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI), 0.5 μg oligo dT (Promega), dNTPs (each at 1 mM; Gibco/BRL), 10 units RNase inhibitor (Promega), and buffer (50 mM Tris–HCl, pH 8.3; 50 mM KCl; 10 mM $MgCl_2$; 0.5 mM spermidine; 10 mM DTT). The reaction mixture was incubated at 42°C for 1 h and then inactivated at 75°C for 10 min.

Quantitation of specific cDNA

Concentrations of lymphokine-specific cDNAs were determined using a quantitative PCR analysis with competitor, or MIMIC, fragments essentially as described in Li *et al.* (1991) and Strehlau *et al.* (1997). MIMIC fragments were heterologous to cDNAs of interest, but contained flanking sequences complementary to the primers specific for these DNAs. Therefore, MIMICs competed with the DNAs of interest for specific primers acting as internal standards in PCR reactions. The MIMIC fragments were obtained using the PCR MIMIC Construction Kit (Clontech). Composite primers (The Great American Gene Company, Ramona, CA) containing sequences complementary to both a nonhomologous internal standard (the *Bam*HI/*Eco*RI fragment of v-erbB cDNA) and the cDNAs of interest (IL-2, β-actin) were used to amplify this nonhomologous competitor fragment and to introduce flanking lymphokine cDNA sequences to the resulting chimeric DNA molecule. For β-actin, the composite primers were 5′-GTG GGG CGC CCC AGG CAC CAC GCA AGT GAA ATC TCC TCC G-3′ and 5′-CTC CTT AAT GTC ACG CAC GAT TTC GGG ACA AGA TAC TCA TCT GC-3′. For IL-2, the composite primers were 5′-ACT CAC CAG GAT GCT CAC ATC GCA AGT GAA ATC TCC TCC G-3′ and 5′-AGG TAA TCC ATC TGT TCA GAT TGA GTC CAT GGG GAG CTT T-3′ (in both cases v-erbB-specific sequences are underlined). For β-actin, the transcript-specific primers were 5′-GTG GGG CGC CCC AGG CAC CA-3′ and 5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′. For IL-2, they were 5′-ACT CAC CAG GAT GCT CAC AT-3′ and 5′-AGG TAA TCC ATC TGT TCA GA-3′.

The concentrations of the competitors were determined using spectrophotometry and verified by gel electrophoresis. Appropriate dilutions of MIMICs were then prepared for use in competitive PCR reactions. To determine the concentration of a specific cDNA, a series of competitive PCR reactions was performed for this cDNA at a constant concentration of the total sample cDNA (0.05 μg/sample) and varying concentrations of the corresponding MIMIC. The amplification products corresponding to the cDNA of interest and the MIMIC were analyzed using agarose gel electrophoresis and identified according to their characteristic sizes: 266 bp for IL-2, 600 bp for IL-2 MIMIC, 548 bp for β-actin, 420 bp for β-actin MIMIC. The ratio between the two products varied, mirroring the changes in the MIMIC concentration. The molar concentration of the cDNA of interest was determined to be equal to the MIMIC concentration at the point of equal intensity of the cDNA band and the MIMIC band. In addition to the sample DNA and MIMIC, the reaction mixture contained 0.5 unit *Taq* DNA polymerase (Promega), oligonucleotide primers specific for the cDNA of interest (0.5 μM each; The Great American Gene Company), dNTPs (0.2 mM each), 1.5 mM $MgCl_2$, and PCR buffer (Promega) in a total volume of 25 μl. The amount of lymphokine-specific cDNAs was normalized to the amount of cDNA for a housekeeping gene, β-actin. The β-actin cDNA was quantified in every sample, and the molar ratios of lymphokine-specific cDNAs to the β-actin cDNA were determined.

Enzyme-linked immunoadsorbent assays (ELISA)

Concentration of secreted IL-2 was measured by sandwich ELISA using capture and detection antibodies from Pharmingen following the manufacturer's recommendations. Briefly, wells of 96-well plates were coated with a capture antibody (rat IgG2a anti-human IL-2 MAb) and then blocked with 1% BSA in PBS. Supernatant samples and IL-2 standards (Eurocetus, Amsterdam, The Netherlands) were diluted with blocking buffer, added to the wells, and incubated at room temperature for 3 h. The plates were then washed, and a biotinylated detection antibody (mouse IgG1 MAb to human IL-2) was added for 1 h. Streptavidin-conjugated horseradish peroxidase (Vector Laboratories, Burlingame, CA) followed by hydrogen peroxide and the chromogenic peroxidase substrate 2,2′-azinibis(3-ethylbenzthiazoline-sulfonic acid) (Sigma) were added to visualize the detection antibody. Enzymatic reactions were stopped with dimethylformamide and SDS, and the product was measured at 405 nm using a V_{max} Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA). The presence of DNA/histone complexes in cytoplasmic cell lysates was analyzed using a similar ELISA assay using a capture mouse anti-

histone H2B MAb and a detection mouse anti-nucleosome MAb as described in Salgame *et al.* (1997).

Electrophoretic mobility shift assays (EMSA)

Extraction of nuclear proteins for EMSA was performed as described in Schreiber *et al.* (1989). Briefly, cells were harvested, washed, and kept in buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF on ice for 15 min to swell. NP-40 was then added to the samples to a final concentration of 0.5% to lyse the cells. Following centrifugation at 13,000 *g* for 15 s at 4°C, the resultant nuclear pellets were extracted with the buffer containing 20 mM HEPES (pH 7.9), 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF. To detect electrophoretic mobility shifts, oligonucleotides corresponding to the consensus NF-κB and AP-1 DNA response elements (5′-AGT TGA GGG GAC TTT CCC AGG C-3′ and 5′-CGC TTG ATG AGT CAG CCG GAA-3′, respectively) and the distal NF-AT site of the human IL-2 promoter/enhancer (5′-CAT GAA ACA GTT TTT CCT CCT TA-3′) along with their mutant forms incapable of binding transcription factors were used (5′-AGT TGA GGC GAC TTT CCC AGG C-3′, 5′-CGC TTG ATG AGT TGG CCG GAA-3′, 5′-TAA GGC TTC CAA ACT GTT TCA TG-3′, respectively). (Mutated nucleotides are underlined; only one strand is shown for each probe.) Oligonucleotides were ^{32}P-labeled using T4 polynucleotide kinase (Promega) in the presence of 10 μCi [γ-^{32}P]ATP (7000 Ci/mmol; ICN, Irvine, CA). The labeled probes were separated from [γ-^{32}P]ATP using Pharmacia Microspin columns. The final concentration and radioactivity of probes was 0.5 ng/μl and 2×10^8 cpm/ml, respectively. Binding was carried out in 20-μl samples containing equal amounts of total nuclear protein (1–3 μg), 0.5 ng ^{32}P-radiolabeled probe, and 1γ poly(dIdC) (Sigma). NF-AT binding assays were carried out in 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, 5% glycerol, and 1 mM DTT. NF-κB binding assays were carried out in 20 mM Tris (pH 7.5), 60 mM KCl, 2 mM EDTA, 4% Ficoll, and 1 mM DTT. AP-1 binding buffer was the same as that for NF-κB plus 5 mM $MgCl_2$. The binding samples were incubated at room temperature for 30 min and separated on a 5% polyacrylamide gel. The gels were dried and probes were visualized by autoradiography. For supershift assays the reactions were carried out in the presence of 2 μg/sample antibodies to the respective transcription factors (Santa Cruz Biotechnology, Santa Cruz, CA) added 30 min prior to labeled probe.

Luciferase reporter assays

To directly assess the activity of IL-2 promoter/enhancer and that of its DNA response elements, we utilized the reporter constructs in which regulatory DNA sequences were inserted upstream of the firefly luciferase gene in pGL3 basic vector (Promega). pIL-2/GL3 contained the nucleotides −326 to +45 of human IL-2 promoter/enhancer. In other reporter plasmids oligomerized binding sites for transcription factors were inserted into the minimal IL-2 promoter (nucleotides −326 to −294 and −77 to +45). pAP-1/GL3, pNF-AT/GL3, and pNF-κB/GL3 contained, respectively, five copies of the metallothionein promoter AP-1 site, three copies of the distal human IL-2 promoter NF-AT site, and three copies of the Igκ chain enhancer NF-κB site. The constructs were kindly provided by Drs. G. Crabtree (Stanford University) and S. Ho (University of California–San Diego). The pRL-0 vector (Promega) encoding luciferase from *Renilla reniformis* but lacking any eukaryotic promoter was used as an internal control to normalize the expression of firefly luciferase for transfection efficiency (Behre *et al.,* 1999). Cells were electroporated in 500 μl of 2×10^7/ml cell suspension in the Opti-MEM medium (Gibco/BRL) at 250 V, 950 μF, in the presence of a reporter construct (50 μg) and the pRL-0 internal control (3 μg). Cells were activated as described above, harvested, and lysed in Passive Lysis Buffer (Promega). Luciferase activity was measured in the lysates using a Dual-Luciferase Assay System (Promega).

Immunoblotting

Immunoblotting methods were described previously (Biesinger *et al.,* 1995; Broker *et al.,* 1993, 1994; Wiese *et al.,* 1996). Briefly, whole cell lysates were treated with SDS–PAGE sample buffer. Then proteins of these lysates or immunoprecipitates were separated by SDS–PAGE and transferred to nitrocellulose (Bio-Rad Laboratories, Richmond, CA), which was then probed with the 238 anti-Ras MAb (Santa Cruz Biotechnology) in blocking buffer. Then protein bands were visualized by enhanced chemiluminescence using an ECL Plus kit (Amersham Pharmacia Biotech).

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